Roles of protein phosphatases in the regulation of neural differentiation of embryonal carcinoma cells

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博士論文

Roles of protein phosphatases in the regulation of neural differentiation of embryonal carcinoma cells.
（プロテインホスファターゼによる胚性腫瘍細胞の神経分化の制御機構）

東北大学大学院医学系研究科内科学系専攻
（分化・発達医学研究部門、発達病態研究分野）

笹原　洋二

SUMMARY

Mouse P19 embryonal carcinoma cells in aggregation culture in the presence of 10^{-6} M retinoic acid (RA) followed by monolayer culture differentiate into nerve and glial cells. In this study, we demonstrated that the neurofilament-L (NF-L) mRNA and protein levels of these cells were enhanced in accordance with their RA-induced neural differentiation. Okadaic acid (OA) treatment of the cells markedly suppressed this differentiation-dependent NF-L gene expression increase and neurite outgrowth of the cells. Similar results were obtained when tautomycin was used instead of OA, suggesting that inhibition of protein phosphatase(s) is involved in the suppression of neural differentiation. OA treatment did not affect the NF-L gene transcription level, determined by the nuclear run-on transcription assay, but it did reduce the stability of both the 3.5- and 2.3-kb NF-L mRNAs. The expression and activity levels of protein phosphatase 2A (PP2A) and 2B (PP2B) but not protein phosphatase 1 (PP1) and protein phosphatase 2C (PP2C) in P19 cells increased in accordance with the enhanced NF-L gene expression. The presence of OA in the culture medium during the course of the neural differentiation caused a reduced PP2A activity but not PP1 and PP2B activities of the cell extracts. On the other hand, both PP1 and PP2B activities but not PP2A activity of cell extracts were suppressed by the addition of Cyclosporin A (CsA) or FK506 in the cultere medium. However, both CsA and FK506 treatments affected neither NF-L gene expression nor neurite outgrowth. These results demonstrate that the OA treatment inhibits the differentiation-dependent increase in NF-L gene expression by destabilizing its mRNAs and suggest that PP2A plays key roles in differentiation-dependent enhanced expression of the NF-L gene and is the point of action of OA.

Embryonal carcinoma (EC) cells have been used extensively as a model system for studying early embryonic development and differentiation. Because these pluripotent cells resemble the inner cell mass of early embryos, they can be differentiated *in vitro* and the derivatives of all three germ layers (mesoderm, endoderm and ectoderm) can be obtained depending on the culture conditions (1-4).

P19 cells, a murine embryonal carcinoma cell line, in aggregation culture with a relatively high concentration of retinoic acid (RA) followed by monolayer culture differentiate into neural cells and this system has been used as a model of neural differentiation (3, 4). Expression of genes related to neural differentiation *in vivo* has also been observed during the process of RA-induced P19 cell differentiation (5-8). A study of signal transmission related to this differentiation revealed that receptor-type protein tyrosine phosphatase α (R-PTPα) participates in the early stage of differentiation (9). However, little is known about the involvement of phosphorylation and dephosphorylation of cellular protein serine and threonine residues in the regulation of such differentiation. We, therefore, were interested in studying the possible functions of major protein serine/threonine phosphatases during the course of P19 cell-differentiation.

The major eukaryotic cellular protein serine/threonine phosphatases have been divided into four classes (PP1, PP2A, PP2B and PP2C) on the basis of their sensitivities to two thermostable proteins (inhibitor-1 and -2) and their divalent cation requirements (10). Recent investigations revealed various physiological roles of each class of protein serine/threonine phosphatases in the regulation of cellular functions (11-14). Okadaic acid (OA) and tautomycin are known to be the inhibitors of PP1, PP2A and PP2B and to be permeable through plasma membranes of cells (15-18). Cyclosporin A (CsA) and FK506, used clinically as immunosuppressants, have been found to form complexes with immunophilines in the cells and inhibit specifically PP2B activity (19, 20). These specific inhibitors of protein phosphatases have provided new tools for exploring the roles of protein phosphatases in the cell culture system.

Neurofilaments (NFs) are intermediate filaments formed from three component proteins, termed NF-L (68 kDa), NF-M (150 kDa) and NF-H (200 kDa), which are encoded by three different genes and expressed specifically in neurons (21-24). PP2A but not PP1 was reported to be able to

remove specifically the phosphate moieties from the NH_2-terminal domain of the NF-L protein *in vitro* (25) and OA treatment caused disruption of the NF network characterized by hyperphosphorylation of NF subunits (26), suggesting that PP2A participates in the regulation of NF-L network assembly.

In this study, we demonstrated that the NF-L expression level was enhanced as neural differentiation of P19 cells progressed and that OA treatment of these cells inhibited this NF-L expression enhancement by reducing the stability of NF-L mRNA. Furthermore, the results suggest that PP2A participates in the mechanism(s) responsible for the differentiation-dependent increase in NF-L expression observed in these cells.

MATERIALS AND METHODS

Materials: [γ-^{32}P]ATP, [α-^{32}P]dCTP and [α-^{32}P]UTP were purchased from DuPont-NEN. OA, tautomycin, all-trans retinoic acid (RA) and the human β-actin cDNA probe were purchased from Wako Pure Chemicals (Osaka, Japan). CsA was from Sandoz (Basel, Switzerland), and FK506 was from Fuzisawa Pharmaceutical (Osaka, Japan). Inhibitor-2 was a gift from Dr. E. Y. C. Lee (University of Miami, FL) and protease inhibitors and calmodulin-agarose were purchased from Sigma (St. Louis, MO). An anti-serum against the catalytic subunit of rat PP2A was raised by immunizing rabbits against C-terminal oligopeptides common to two distinct isoforms of the catalytic subunit of PP2A (Cα and Cβ) (27), and the anti-serum against the rat PP2Cα was raised by immunizing rabbits the recombinant whole PP2Cα protein. The anti-rabbit PP1α catalytic subunit polyclonal antibody was purchased from UBI (New York, NY) and the monoclonal antibodies against the catalytic and regulatory subunits of PP2B were gifts from Dr. J. H. Wang (University of Manitoba, Canada). The anti-mouse neurofilament-L monoclonal antibody was purchased from Oncogene Science (New York, NY), the anti-rabbit immunoglobulin G (IgG)/alkaline phosphatase-conjugate antibody was from Promega (Madison, WI), the anti-mouse IgG/horseradish peroxidase-conjugate antibody was from Bio-Rad, and pCR™II was purchased from Invitrogen (San Diego, CA). Myosin light chain and myosin light chain kinase were gifts from Dr.Masaaki Ito (Mie University, Japan).

Cells and Culture: P19 EC cells were obtained from ATCC (Rockville, MD) and cultured in bicarbonate-buffered α-modified Eagle's medium supplemented with 10% (v/v) fetal calf serum under a humidified 5% (v/v) CO_2 in air atmosphere at 37 ℃. In order to induce neural differentiation, the cells were cultured on bacterial-grade dishes to form aggregates, termed embryoid bodies, for 4 days in the presence or absence of 10^{-6} M RA, then replated on tissue culture-grade dishes and cultured for two more days without RA (4). OA (10 nM), tautomycin (50 nM), CsA

(1 μg/ml) or FK506 (100 ng/ml) was added to the medium for the required times.

Northern Blot Analysis: The total RNA was isolated from the cells, after incubation for the required times, by the acid guanidium thiocyanate-phenol-chloroform method. 10-μg aliquots of denatured RNA were electrophoresed, transferred onto Hybond N^+ membranes (Amersham, Corp.) and Northern hybridization was carried out, as described previously (28). The DNA probes for PPs used were as follows: the 259-base pairs (bp) cDNA fragment unique to the α isoform of the catalytic subunit of rat PP1 prepared by the polymerase chain reaction (PCR) using the upstream (5'-CTGTG GCGAGTTTGACAACGCTGCC-3') and downstream (5'-TCATGCTGCCATGGGTCACACTGG CCTCTCA-3') primers (29); the 1.8-kilobase pairs (kbp) *Eco* RI fragment of the rat PP2A catalytic subunit (Cα isoform) cDNA (30) and the 360-bp *Pst* I fragment of the rat PP2B catalytic subunit (Aβ isoform) cDNA (31); the 0.8kbp *Eco* RI fragment of the rat PP2Cα (32) and the 0.6kbp *Pst* I fragment of the mouse PP2Cβ (28). The DNA probe for mouse NF-L (720 bp) was prepared by the polymerase chain reaction using the upstream (5'-ACAAGCGGCGCTATGTGGAG-3') and downstream (5'-TCGGGCTTGGAGGACACGTC-3') primers (21). The 190-bp DNA probe for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was prepared by the polymerase chain reaction using the upstream (5'-TGGCATTGTGGAAGGGCTCATGAC-3') and downstream (5'-ATGCCAGTGAGCTTCCCGTTCAGC-3') primers (33). All these fragments were ^{32}P-labeled by the random primer labeling method and used as probes. Radioactivity was measured by an auto-image analyzer (BAS 2000, Fuji, Japan) or autoradiography using Kodak X-ray films.

Preparation of Cell Extracts and Immunoblotting: The cells were washed twice with isotonic saline and suspended in buffer C (10 mM Tris-HCl (pH 6.9), 50 mM NaCl, 5 mM β-mercaptoethanol, 0.1 mM EDTA, 1mM EGTA and 2% (v/v) glycerol) containing protease inhibitors (2 μg/ml pepstatin A, 2 μg/ml antipain, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 0.1mM N α-tosyl-L-phenylalanine

chloromethyl ketone, 0.1mM N α-*p*-tosyl-L-lysine-chloromethyl ketone, 0.1 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride) and sonicated on ice. Each suspension was centrifuged at 10,000 x g for 10 min and the resulting supernatant was used as the cell extract. 10-µg aliquots of each extract were subjected to SDS-polyacrylamide gel electrophoresis, as described previously (34), electrotransferred onto nitrocellulose membranes (Schleicher & Schuell), and immunostained with specific antibodies, as described previously (28), and immunoreactivity was detected using color development system (28). Protein concentrations were determined using the method described by Bradford (35) with bovine serum albumin as the standard.

Assay of PP Activity: PP activity was assayed by measuring the amount of [^{32}P]phosphate released from [^{32}P]histone, [^{32}P]casein or [^{32}P]myosine light chain, which were prepared with cyclic AMP-dependent protein kinase, casein kinase II and myosine light chain kinase, respectively, as described previously (10, 36, 37). The PP1 activity assay was performed in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 nM OA, 1mM $MnCl_2$, 60 µM (alkaline labile phosphate) [^{32}P]casein and the required cell extract in the presence or absence of 0.2 µM inhibitor-2. The PP1 activity was defined as the inhibitor-2-sensitive PP activity (activity in the absence of inhibitor-2 minus that in its presence). The PP2A activity assay was performed in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2 µM inhibitor-2, 60 µM (alkaline labile phosphate) [^{32}P]histone and the required cell extract in the presence or absence of 1 nM OA. The PP2A activity was defined as the OA-sensitive activity (activity in the absence of OA minus that in its presence). One unit of PP activity was defined as the amount of enzyme that catalyzed the release of 1 nmol phosphate per min. at 30 ℃. The PP2B activity of cell extracts was assayed after purifying PP2B with calmodulin-agarose affinity column. The column chromatography of cell extracts was performed as described previously (38). The purified PP2B fraction was essentially free of PP1, PP2A and PP2C activities. The PP2B avtivity assay was performed in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mMEGTA, 0.2 µM inhibitor-2, 0.5 µM OA, 1 mM

$MnCl_2$, 2 mM $CaCl_2$, 3 μM calmodulin, 60 μM (alkaline labile phosphate) [^{32}P]myosin light chain and purified PP2B fractions. The PP2C activity was measured in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mMEGTA, 0.2 μM inhibitor-2, 1μM OA, 10 mM $MgCl_2$, and 60 μM (alkaline labile phosphate) [^{32}P]histone.

Nuclear Run-on Transcription Assay: Nuclear preparation was perfomed as described previously (39), except that 10 nM OA was present in the Nonidet P-40 lysis buffer. 10-μg aliquots of mouse NF-L cDNA fragment, prepared by the polymerase chain reaction, as described above, were subcloned into pCR™II. pCR™II, with or without the insert, was linearized by *Bam*HI digestion, denatured, and fixed on Hybond N^+ membranes with a Dot-blot apparatus (Advantec, Japan). 10-μg aliquots of the 440-bp fragment of human β-actin cDNA and the 1.2-kbp fragment of chicken GAPDH cDNA (40) were also fixed on the membranes. The run-on products from the same number of nuclei (1.0 x 10^7 nuclei) labeled with [α-^{32}P]UTP were hybridized with the cDNA probes on the membranes at 45 ℃ for 3 days. Then the membranes were washed until no detectable nonspecific radioactivity of the spot of pCR™II without an insert remained (Fig.8).

Analysis of mRNA stability: The stability of NF-L mRNA was analyzed as described previously (24). Neural-differentiated P19 cells were treated with 10μg/ml actinomycin D in the presence or absence of 10nM OA, and harvested at 0, 2, 4 and 6h. after the actinomycin D addition. Their cytoplasmic RNA fractions were extracted and subjected to Northern blot analysis using the NF-L and GAPDH cDNA fragments as probes. The intensity of the radioactive bands was measured by an auto-image analyzer.

RESULTS

Expression of Neurofilament-L during the Course of P19 Cell Neural Differentiation.

First we determined whether the expression level of neurofilament-L (NF-L), a marker of neural differentiation, altered during the course of RA-induced differentiation of P19 cells into neurons and glias. The morphological changes during the course of neural differentiation are shown in Fig. 1. When the P19 cells were cultured on bacterial-grade dishes, cell aggregates termed embryoid bodies were formed about 24 h after seeding both in the presence and absence of 10^{-6} M RA. Neurite outgrowth was observed about 48 h after replating onto tissue culture-grade dishes (monolayer culture) in the absence of RA only when the preceding aggregation culture had been performed in the presence of RA, essentially confirming the previous report (Fig.1, *B* and *C*)(4). The amount of cellular NF-L protein in the aggregation culture increased markedly only when RA was present and this elevated level was maintained in the subsequent monolayer culture (Fig. 2*A*). The NF-L mRNA level was found to increase in accordance with the enhanced NF-L protein level (Fig. 2*D*).

PP1, PP2A , PP2B and PP2C Expression during the Course of P19 Cell Differentiation.

In order to determine whether the expression levels of PPs alter during the course of the neural differentiation of P19 cells, the levels of mRNA, protein and activity of four major PPs (PP1, PP2A, PP2B and PP2C) were studied. Neither the intensity of the mRNA signal of the PP1α catalytic subunit, PP1α protein level, nor the total PP1 activity level in the cell extracts changed significantly during the course of differentiation (Figs. 3, *A*, *B*, and *C*).

In contrast, the level of the mRNA signal of the PP2A catalytic subunit (Cα) increased in the aggregation culture only in the presence of RA, and this high level was maintained during the following monolayer culture (Fig. 4*A*). Immunoblotting showed that the alteration in the pattern of the protein level of the PP2A catalytic subunits (mixture of PP2A · Cα and PP2A · Cβ proteins)

during the course of differentiation was similar to that of the mRNA levels (Fig. 4*B*). In parallel with the mRNA and PP2A catalytic subunit protein levels, the total PP2A activity increased (1.5-fold) in the aggregation culture in the presence of RA and increased further (2.2-fold) in the subsequent monolayer culture (Fig.4*C*), whereas no alterations of PP2A expression were observed when the cells were cultured in the absence of RA.

The mRNA and protein levels of the PP2B catalytic subunit also increased when aggregation culture was performed in the presence but not the absence of RA and the expression level increased further in the subsequent monolayer culture (Figs. 5, *A* and *B*). In contrast with PP2B catalytic subunit, the presence of substantial amount of PP2B regulatory subunit protein was observed in the control P19 cell extracts, but the expression level of the regulatory subunit was also enhanced during the course of the differentiation process (Fig. 5*B*). In parallel with the enhanced expression of the catalytic and regulatory subunits of PP2B, the PP2B activity of the cell extract increased in the aggregation culture in the presence of RA, and the activity level reached 4.3-fold above control in the subsequent monolayer culture. However, no increase in the activity level was observed when the cells were not treated with RA (Fig. 5*C*).

With regard to the expression of PP2C, the size of the major PP2Cβ isoform was 2.8kb (28) in the untreated control cells, and an enhanced level of an additional 3.5kb mRNA signal encoding PP2Cβ-1 (28) was observed on Northern analysis (Fig. 6*A*). However, the expression levels of PP2Cα mRNA and PP2Cα protein did not alter significantly (Fig. 6, *A* and *B*). In addition, the total activity of PP2C did not alter during the course of the differentiation (Fig. 6*C*). Therefore, the significance of the differentiation-dependent increase in the 3.5kb mRNA signal is not known.

Effect of Protein Phosphatase Inhibitors on Neurite Outgrowth and NF-L Expression.

In order to determine whether the enhanced expression levels of PP2A and PP2B are related to the regulation of P19 cell differentiation, the effects of OA and tautomycin, inhibitors of PP1, PP2A and PP2B, and CsA and FK 506, specific inhibitors of PP2B, were studied. When 10 nM OA was present in the culture medium throughout the differentiation process, neurite outgrowth and the

mRNA (both 2.3- and 3.5-kilobases mRNAs) and protein levels of NF-L were suppressed markedly on day 6 (Figs. 1*D*, 2*B* and 2*D*). OA at 10 nM had no effect on the viability of P19 cells (Fig. 7). Treatment with 5 nM OA had a considerably less marked inhibitory effect on neurite outgrowth and NF-L expression (data not shown). The presence of 50 nM tautomycin in the culture medium during the course of differentiation partially suppressed the neurite outgrowth and NF-L expression (Figs. 1*E*, 2*C*, and 2*E*), but this concentration of tautomycin did not affect the viability of the cells. The presence of 10 nM OA during the last 2 days (days 4-6) only of the course of differentiation was enough to suppress NF-L expression (Figs. 2, *B* and *D*). However, no decrease in the NF-L level was observed when the cells were treated with OA for the first (days 0-2) or second (days 2-4) 48 h only of the 6-day course of P19 cell neural differentiation. In accordance with the effect of OA treatment on NF-L expression, neurite outgrowth was partially inhibited by treating the cells with OA during days 4-6, but not during days 0-2 or 2-4 (data not shown).

The addition of 1 μg/ml CsA to the culture medium throughout the differentiation process affected neither neurite outgrowth nor the NF-L expression level (Figs. 1*F* and 2*F*). The addition of 100 ng/ml FK506 did not affect the neurite outgrowth, but it caused an enhanced expression of NF-L (Figs.1*G* and 2*F*).

Effect of OA Treatment on NF-L Gene Transcription and NF-L mRNA Stability .

In order to determine the mechanism by which OA treatment reduced the NF-L mRNA levels, we performed the nuclear run-on transcription assay using nuclei isolated from neurally differentiated P19 cells (day 6) treated with or without 10 nM OA (Fig. 8). The results showed that there were no significant differences between the NF-L gene transcriptional levels of cells treated with and without OA.

Next, the effect of OA treatment on NF-L mRNA stability was studied. The cytoplasmic NF-L mRNA level of differentiated cells treated with OA was compared with that of untreated cells after incubating both sets of cells with actinomycin D for 0, 2, 4 and 6 h (Fig. 9). OA treatment enhanced degradation of both 3.5- and 2.3-kilobase NF-L mRNAs, whereas GAPDH mRNA stability was affected little by OA treatment.

Effects of OA, CsA and FK506 Addition during the Course of Differentiation on the PP1, PP2A and PP2B Activities

It has been reported that endogenous PP2B activity of cell extracts was suppressed when the enzyme assay was performed following the incubation of mammalian cells in the presence of CsA (41) . Therefore, we tested whether the treatment of P19 cells with the protein phosphatase inhibitors in the course of neural differentiation affected the protein phosphatsase activities of the cell extract. The PP1, PP2A and PP2B activities of cell extracts were determined after culturing the cells in the presence of OA, CsA or FK506 during the 6-day course of the neural differentiation (Fig. 10). The PP1 activity decreased 29 % and 38 % when the cells were treated with CsA and FK506, respectively, compared with that of the control cells without treatments, but the activity was little affected by the OA treatment (Fig. 10*A*). The PP2A activity decreased 68 % by the OA treatment of the cells, whereas the CsA or FK506 treatment affected the enzyme activity very little (Fig. 10*B*). On the other hand, the PP2B activity decreased 90% and 77% by the CsA and FK506 treatments, respectively , whereas the 10nM OA treatment did not significantly affect the PP2B activity in the cell extracts (Fig. 10*C*).

DISCUSSION

In this study we demonstrated that adding OA or tautomycin to the culture medium morphologically suppressed RA-induced neural differentiation of P19 cells. We demonstrated also that NF-L gene expression increased in accordance with P19 cell neural differentiation and both OA and tautomycin treatments inhibited NF-L gene expression. The evidence that both OA and tautomycin showed similar inhibitory effcts on the neural differentiaion of P19 cells suggests that the inhibition was operated through the suppression of protein phosphatase activities by these agents.

The inhibition by OA treatment was due primarily to reduced NF-L mRNA stability. As the GAPDH mRNA level was hardly affected by OA treatment, the reduced NF-L mRNA stability did not appear to be a nonspecific effect of OA (Fig. 2D). Regulation of NF-L gene expression in rat PC12 cells by posttranscriptional modification has been reported (42), and in this cell system, nerve growth factor increased NF-L mRNA levels by stabilizing the mRNAs.

We found that expression and cellular activities of PP2A and PP2B but not PP1 and PP2C increased in parallel with the NF-L expression level of P19 cells (Figs. 3-6). Because adding CsA or FK506, specific inhibitors of PP2B, to the culture medium did not inhibit NF-L expression, PP2B was unlikely to be responsible for the differentiation-dependent increase in NF-L expression (Figs. 1 and 2). In this context, FK506 treatment was reported to rather stimulate nerve growth factor-induced neural differentiation of PC12 cells (43). OA inhibited NF-L expression only when high levels of PP2A activity were maintained and a relatively low concentration of OA (10 nM) was required for this inhibition (Figs. 2, B and D). OA addition in the culture medium inhibited the PP2A activity but not the PP1 activity of the cell extracts harvested at day 6 of the cell differentiation process. In addition, both CsA and FK506 treatments, which did not suppress the NF-L expression, were found to inhibit not only the PP2B activity but also the PP1 activity of the cell extracts (Fig.10). These lines of evidence suggests strongly that of the three major OA-sensitive protein phosphatases PP2A but not PP1 or PP2B is responsible for the differentiation-dependent increase in NF-L expression and is the point of the action of OA. The inhibition of PP1 activity by CsA or FK506 treatment was presumably through inhibition of PP2B by these agents, because it has been reported

that a CsA- or FK506-induced decrease in the PP2B activity in nerve cells contributed to maintenance of the high phosphorylation level of protein phosphatase inhibitor-1, which as a result caused decrease in the PP1 activity of the cells (13).

It is generally accepted that PP2A is present in various oligomeric forms in mammalian cells. The activity of the free catalytic subunit changes considerably when it recombines with the regulatory subunits (44) and PP2A activity can be regulated by post-translational modifications (45, 46). Therefore, it is unlikely that the differentiation-induced PP2A activity increase was caused merely by enhanced expression of the catalytic subunit of PP2A. The possibility that some post-translational modification of PP2A induced by P19 cell differentiation contributed to the increased PP2A activity should be considered.

Various information regarding the involvement of protein phosphorylation in the regulation of mRNA stabilization has been obtained. In LLC-PK1 cells, down-regulation of protein kinase C was found to stabilize urokinase-type plasminogen activator mRNA (47), and CsA treatment of mast cell tumor lines was reported to destabilize interleukin-3 mRNA through the mechanism involved the 3'-untranslated region (3'-UTR) (48), suggesting that PP2B enhances mRNA stability. However, in contrast to its destabilizing effect on NF-L mRNA observed in this study (Fig. 9), OA treatment was found to increase the stability of nerve growth factor mRNAs in primary cultures of cortical astrocytes (49). Such conflicting evidence suggests that the stability of mRNAs is controlled by various signal transfer systems and phosphorylation of cellular proteins results in distinct effects that depend on the particular mRNA species and/or the signal transfer system employed. Schwartz *et al.* (50) reported recently that 3'-UTR of NF-L mRNA may contain determinants that regulate the stability of NF-L mRNA since studies of transfected P19 cells showed 3'-UTR deletion led to a severalfold stabilization of NF-L mRNA, providing the possibility that OA-sensitive dephosphorylation of cellular proteins is involved in the inhibition of destabilizing function of 3'-UTR of NF-L mRNA . In this context, they also detected two different proteins that binded to the 3'-UTR of NF-L mRNA, speculating that PP2A might be one of the regulatory molecules of the function of these RNA-binding proteins.

OA treatment of rat dorsal root ganglion neurons was reported to cause NF-network disruption characterized by hyperphosphorylation of NF-subunits (26), and the amino-terminal domain of NF-L was preferentially dephosphorylated by the catalytic subunit of PP2A but not that of PP1 *in vitro* (25). Therefore, the differentiation-dependent increase in PP2A expression in P19 cells may contribute not only to enhanced NF-L expression but also to NF-L dephosphorylation, thereby inducing assembly of the NF network .

It is not known what type(s) of signal is/are required to enhance the expression levels of the PP2A and PP2B catalytic subunits in P19 cells. In this context, den Hertog *et al.* (9) reported that R-PTPα participated in signal transmission related to P19 cell aggregation. They found that the R-PTPα expression level was enhanced when P19 cells in monolayer culture were transferred to aggregation culture and that P19 cells harboring the R-PTPα expression vector could be differentiated into neurons in a RA-dependent manner, even in the monolayer culture. Their results also demonstrated that endogenous pp $60^{c\text{-}src}$ is activated by a high R-PTPα expression level. Therefore, in order to clarify the mechanism(s) responsible for the differentiation-dependent increases in PP2A and PP2B expression, it is important to determine whether high expression levels of these enzyme molecules are related to R-PTPα/pp $60^{c\text{-}src}$-dependent signal transmission.

In this paper, we describe that PP2A activity is a potent regulator of the neural differentiation of P19 cells, and suppresses the differentiation-dependent expression of NF-L gene by post-transcriptional modification. Recently, some physiological roles of phosphatases were reported in mature neural cells, including the dephosphorylation of neuron-specific proteins, the regulation of the Ca^{2+} / calmodulin-dependent kinase II activity in neural cells, and the long-term depression (LTD) of rat hippocampal neurons (12-14). However, little is known about the involvement of phosphatases in the regulation of the neural differentiation. We expect that this P19-neural differentiation system will be a usuful model for the assay of the neural cell function, and our results will be a clue to exploring the roles of phosphatases in the neural differentiation systems.

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FOOTNOTES

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Abbreviations: EC, embryonal carcinoma; RA, retinoic acid; R-PTPα, receptor-type protein phosphatase α; NF, neurofilament; OA, okadaic acid; CsA, cyclosporin A; PP, protein phosphatase; kDa, kilodalton; IgG, immunoglobulin G; kbp, kilobase pair(s); PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s).

LEGENDS TO FIGURES

Figure 1. Photomicrographs showing the effects of protein phosphatase inhibitors on neurite outgrowth during P19 cell neural differentiation. Undifferentiated P19 cells (day 0) (*A*) were cultured on bacterial-grade dishes with (*C-G*) or without (*B*) RA for 4 days and subsequently cultured on culture-grade dishes without RA for 2 days. The photographs were taken on days 0 (*A*) and day 6 (*B-G*). For cell treatment, 10 nM OA (*D*), 50 nM Tautomycin (*E*), 1 μg/ml CsA (*F*) or 100ng/ml FK506 (*G*) was present in the medium from day 0 throughout the differentiation process, while no phosphatase inhibitors were present in *B* and *C*.

Figure 2. Effects of protein phosphatase inhibitors on NF-L expression. *A*, cells cultured in the absence (*lanes 1-3*) or presence (*lanes 4* and *5*) of RA were harvested at the indicated times (*lane 1*, day 0; *lanes 2* and *4*, day 4; *lanes 3* and *5*, day 6) and the cell extracts were subjected to immunostaining using the anti-NF-L antibody as the first antibody. *B* and *D*, cells were cultured in the absence (*lane 2*) or presence of 10 nM OA during days 0-2 (*lane 3*), 2-4 (*lane 4*), 4-6 (*lane 5*) and 0-6 (*lane 6*) of the course of RA-induced differentiation. *Lane 1*, the cells were cultured in the absence of RA and OA. In all cases, the cells were harvested on day 6. Immunoblotting was performed using the anti-NF-L antibody as the first antibody (*B*) and Northern blot analysis of NF-L and GAPDH mRNAs was performed using the total RNA fractions of the cells (*D*). *C* and *E*, cells were cultured in the absence (*lane 1*) or presence (*lanes 2* and *3*) of 10^{-6} M RA for the first 4 days on bacterial-grade dishes and then on culture-grade dishes in the absence of RA for 2 days. In *lane 3*, 50 nM tautomycin was present throughout the course, and the cells were harvested on day 6 in all cases. Immunoblotting of NF-L (*C*) and Northern blot analysis of NF-L and GAPDH mRNAs (*E*) were performed as described in the legends to *B* and *D*, respectively. *F*, cells were cultured in the absence (*lane 1*) or presence (*lanes 2-4*) of 10^{-6} M RA for the first 4 days on bacterial-grade dishes and then on culture-grade dishes in the absence of RA for 2 days. Throughout the course of differentiation, 1 μg/ml CsA (*lane 3*) or 100 ng/ml FK506 (*lane 4*) was present in the culture medium.

The extracts of the cells harvested on day 6 were immunoblotted using the anti-NF-L antibody as the first antibody. *kb*, kilobases.

Figure 3. PP1 expression level during the course of P19 cell neural differentiation.
A, the total RNA fractions were extracted from the cells at the indicated times during the course of P19 cell neural diffrentiation and Northern hybridization was performed using the PP1α and GAPDH cDNA fragments as probes. *B*, the extracts of cells harvested at the indicated times during the course of neural differentiation were immunoblotted using the anti-PP1α antibody as the first antibody. *C*, PP1 activities of cell extracts harvested at the indicated times during the course of differentiation were determined, as described under "Materials and Methods" using [^{32}P]casein as the substrate. The results represent the mean of three experiments ± standard error of the mean.

Figure 4. PP2A expression level during the course of P19 cell neural differentiation.
A, Northern hybridization of the total RNA fractions described in the legend to Fig. 3*A* was performed using the cDNA fragments specific for the α isoform of the rat PP2A catalytic subunit and GAPDH as probes. *B*, the cell extracts described in the legends to Figs. 2*A* and 3*B* were immunoblotted using the rabbit anti-serum raised against the oligopeptide of the PP2A catalytic subunit as the first antibody. *C*, the cell extracts described in the legend to Fig. 3*C* were used for the PP2A activity assay with [^{32}P]histone as the substrate. The results represent the mean of three experiments ± standard error of the mean.

Figure 5. PP2B expression level during the course of P19 cell neural differentiation.
A, Northern hybridization of the total RNA fractions described in the legend to Fig. 3*A* was performed using cDNA fragments of the β isoform of the rat PP2B catalytic subunit and GAPDH as probes. *B*, the cell extracts described in the legends to Figs. 2*A* and 3*B* were immunoblotted using mouse monoclonal antibodies raised against the catalytic and regulatory subunits of rat PP2B as

the first antibodies. *C*, the same amounts of proteins in the affinity-purified PP2B fractions from cell extracts were subjected to the PP2B activity assay with [^{32}P]myosin light chain as the substrate as described under "Materials and Methods".

Figure 6. PP2C expression level during the course of P19 cell neural differentiation. *A*, Northern hybridization of the total RNA fractions of undifferentiated P19 cells (day 0) and neural differentiated cells (RA+, day6) was performed using the cDNA fragments specific for the rat PP2Cα , mouse PP2Cβ and GAPDH as probes. *B*, the cell extracts described above were immunoblotted using the polyclonal antibody AB103 raised against the rat PP2Cα recombimant protein as the first antibody. *C*, the cell extracts described in the legend to Fig. 3*C* were used for the PP2C activity assay with [^{32}P]histone as the substrate. The results represent the mean of three experiments ± standard error of the mean.

Figure 7. Effect of OA treatment on the growth rate of undifferentiated P19 cells. P19 cells (2×10^4 cells/dish) were cultured in monolayer in the absence or presence of 5 or 10 nM OA, and the numbers of viable cells were counted by trypan blue exclusion method at the indicated times after seeding. The results represent the mean of three independent experiments ± standard error of the mean.

Figure 8. Effect of OA treatment on NF-L gene transcription in P19 cells. *A*, the cells were cultured in the presence of 10^{-6} M RA with (*lane 1*) or without (*lane 2*) 10 nM OA for the first 4 days on bacterial-grade dishes and then cultured in the absence of RA with (*lane 1*) or without (*lane 2*) OA on culture-grade dishes for 2 days. The cells were harvested on day 6, cell nuclei were prepared and the nuclear run-on transcription assay was performed, as described under "Materials and Methods". Transcriptional levels of GAPDH and β-actin genes are also depicted. *B*, the radioactivity of each spot was quantitated by an auto-image analyzer (BAS 2000), and the mean of two independent

experiments ± standard error of the mean was represented.

Figure 9. Effect of OA treatment on NF-L mRNA stability. After RA-induced differentiation, P19 cells were incubated with 10 μg/ml actinomycin D in the presence or absence of 10 nM OA, and harvested at the indicated times, and their cytoplasmic RNA fractions were extracted. 20-μg aliquots of RNA were subjected to Northern blot analysis using the NF-L and GAPDH cDNA fragments as probes. The intensities of the radioactive bands of 3.5- and 2.3-kilobase(kb) NF-L and GAPDH mRNAs were measured by an auto-image analyzer. The results represent the mean of three experiments ± standard error of the mean.

Figure 10. Effects of OA, CsA and FK506 treatments on PP activities. PP1 (*A*), PP2A (*B*) and PP2B(*C*) activities of cell extracts were determined as described under "Materials and Methods" after culturing the cells in the absence or presence of 10 nM OA, 1μg/ml CsA or 100 ng/ml FK506 during the 6-day course of the neural differentiation. Each activity is expressed as percentage of the activity of the control cells that were not treated with protein phophatase inhibitors.The results represent the mean of two experiments ± standard error of the mean.

Fig.1

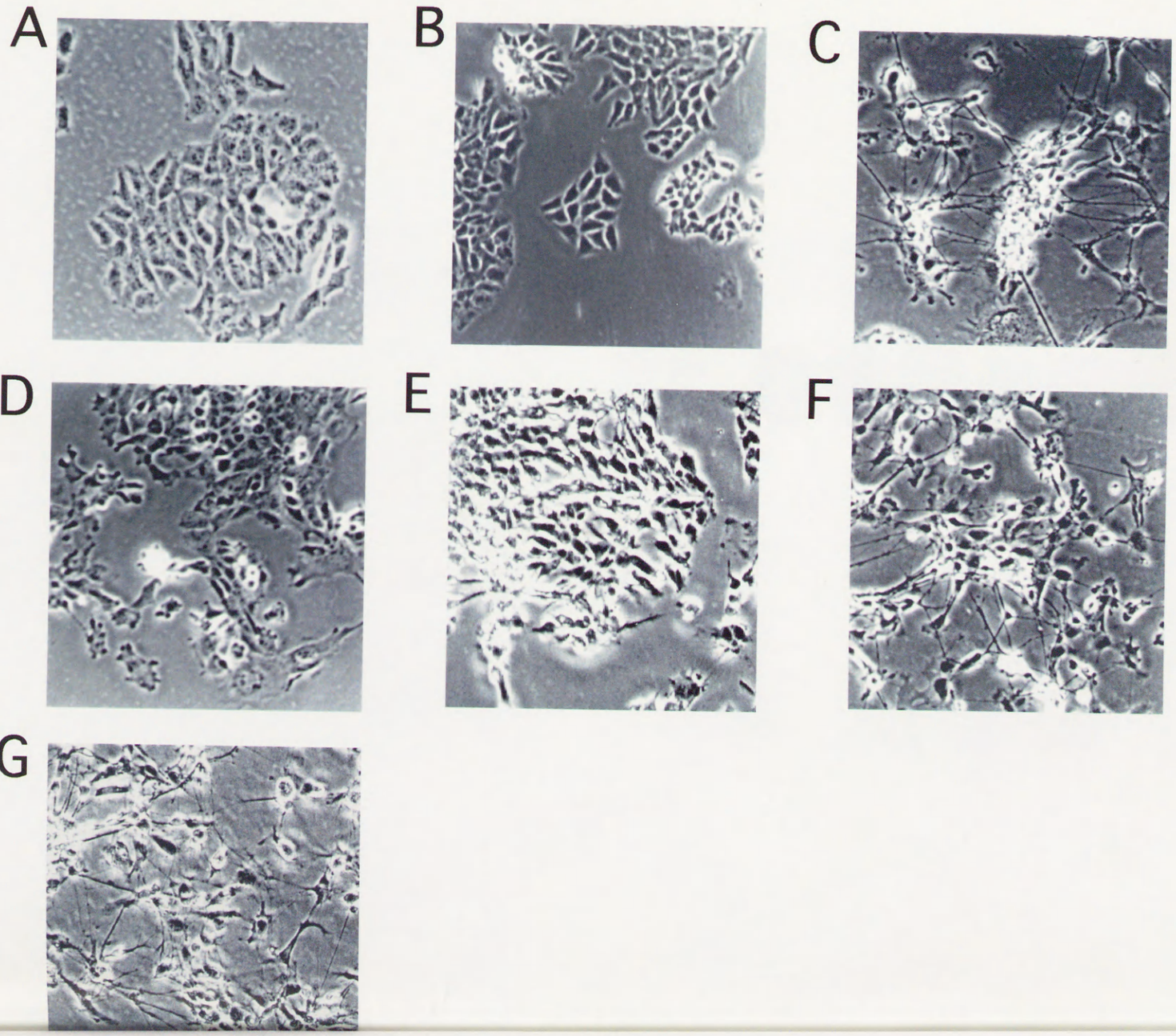


Fig.2

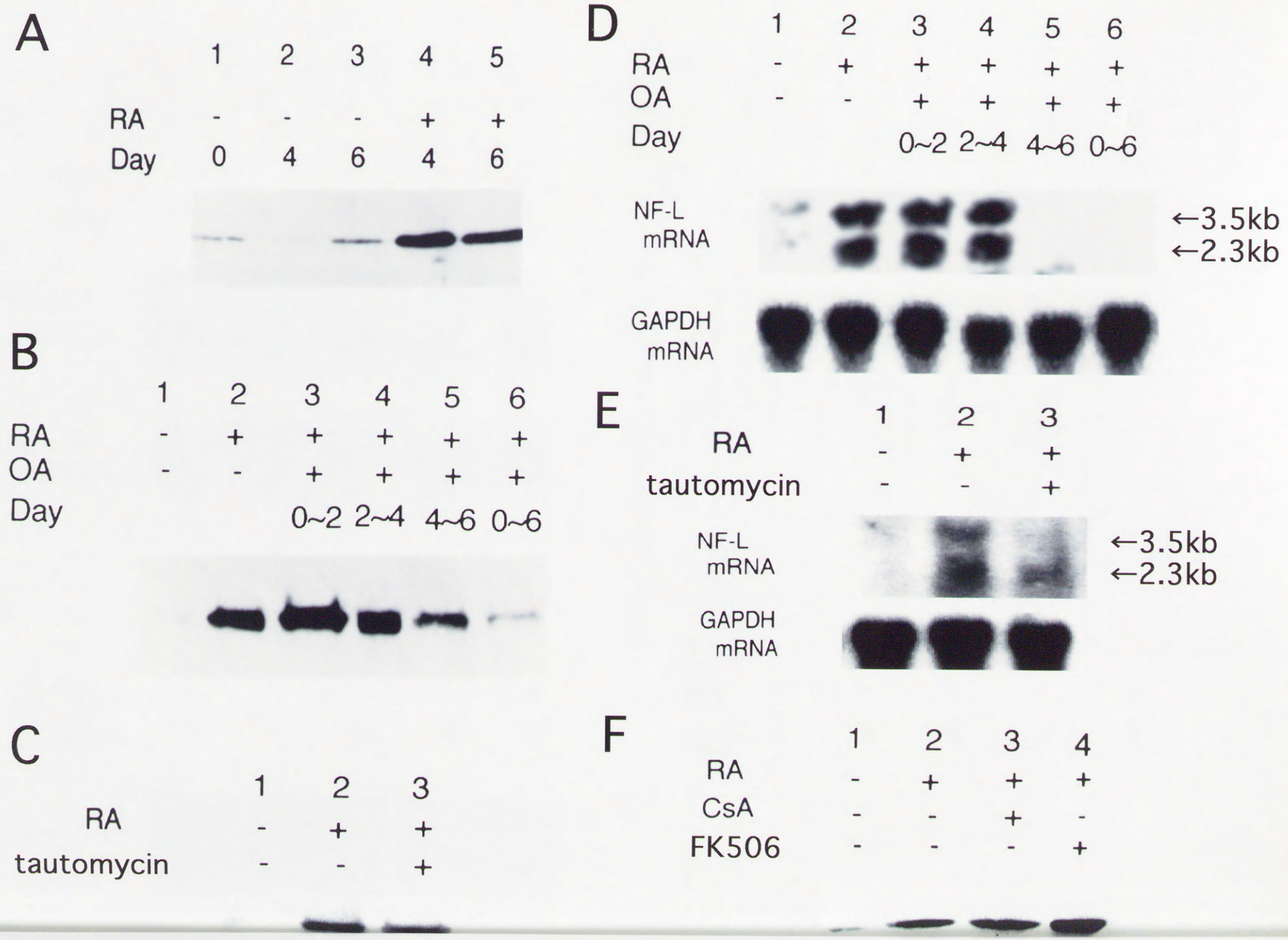

A
1 2 3 4 5
RA - - - + +
Day 0 4 6 4 6
B
1 2 3 4 5 6
RA - + + + + +
OA - - + + + +
Day 0~2 2~4 4~6 0~6
C
1 2 3
RA - + +
tautomycin - - +
D
1 2 3 4 5 6
RA - + + + + +
OA - - + + + +
Day 0~2 2~4 4~6 0~6
NF-L mRNA
←3.5kb
←2.3kb
GAPDH mRNA
E
1 2 3
RA - + +
tautomycin - - +
NF-L mRNA
←3.5kb
←2.3kb
GAPDH mRNA
F
1 2 3 4
RA - + + +
CsA - - + -
FK506 - - - +

Fig.3

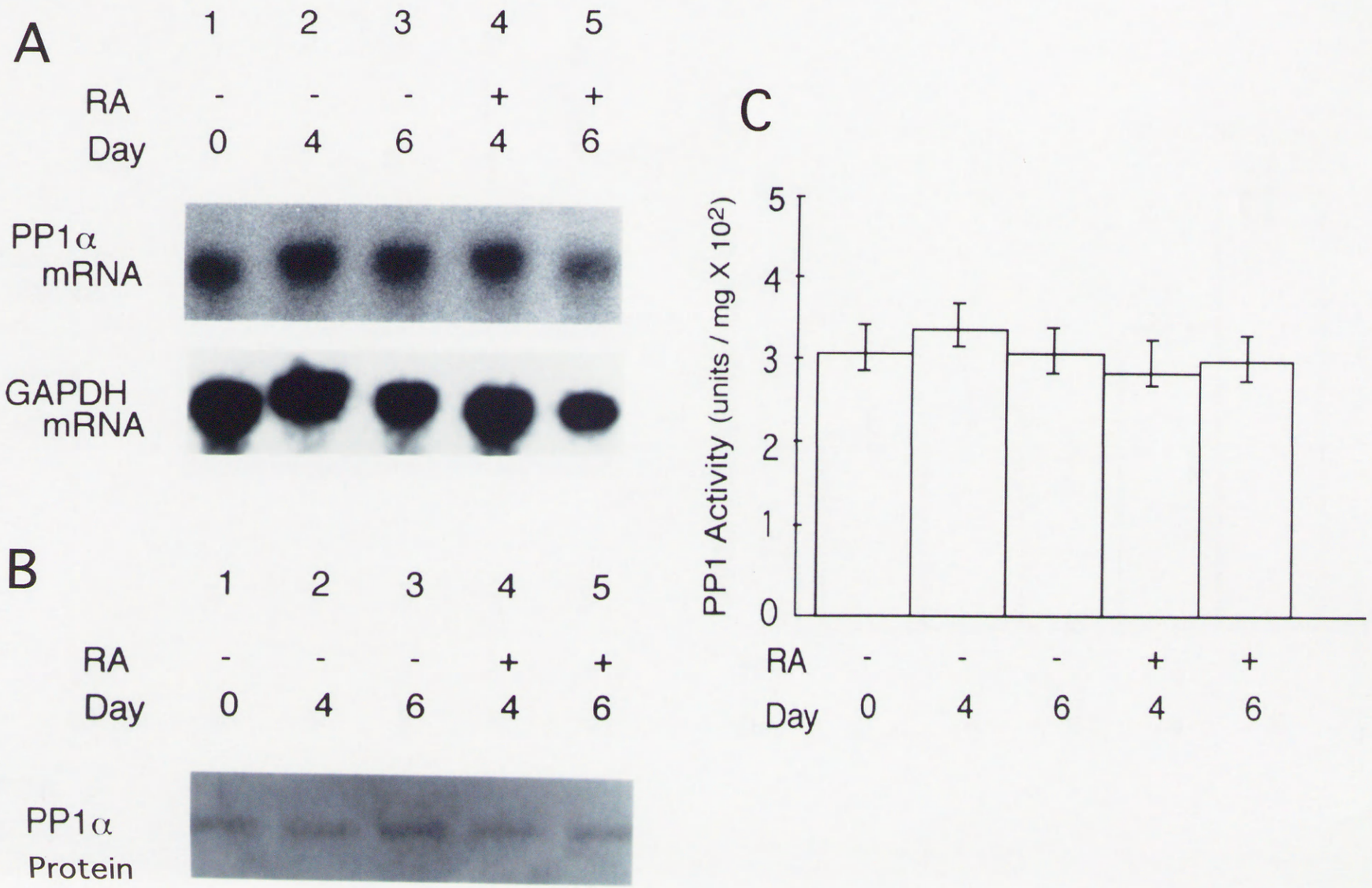

A
1 2 3 4 5
RA - - - + +
Day 0 4 6 4 6
PP1α mRNA
GAPDH mRNA
B
1 2 3 4 5
RA - - - + +
Day 0 4 6 4 6
PP1α Protein
C
PP1 Activity (units / mg X 10²)
0 1 2 3 4 5
RA - - - + +
Day 0 4 6 4 6

Fig.4

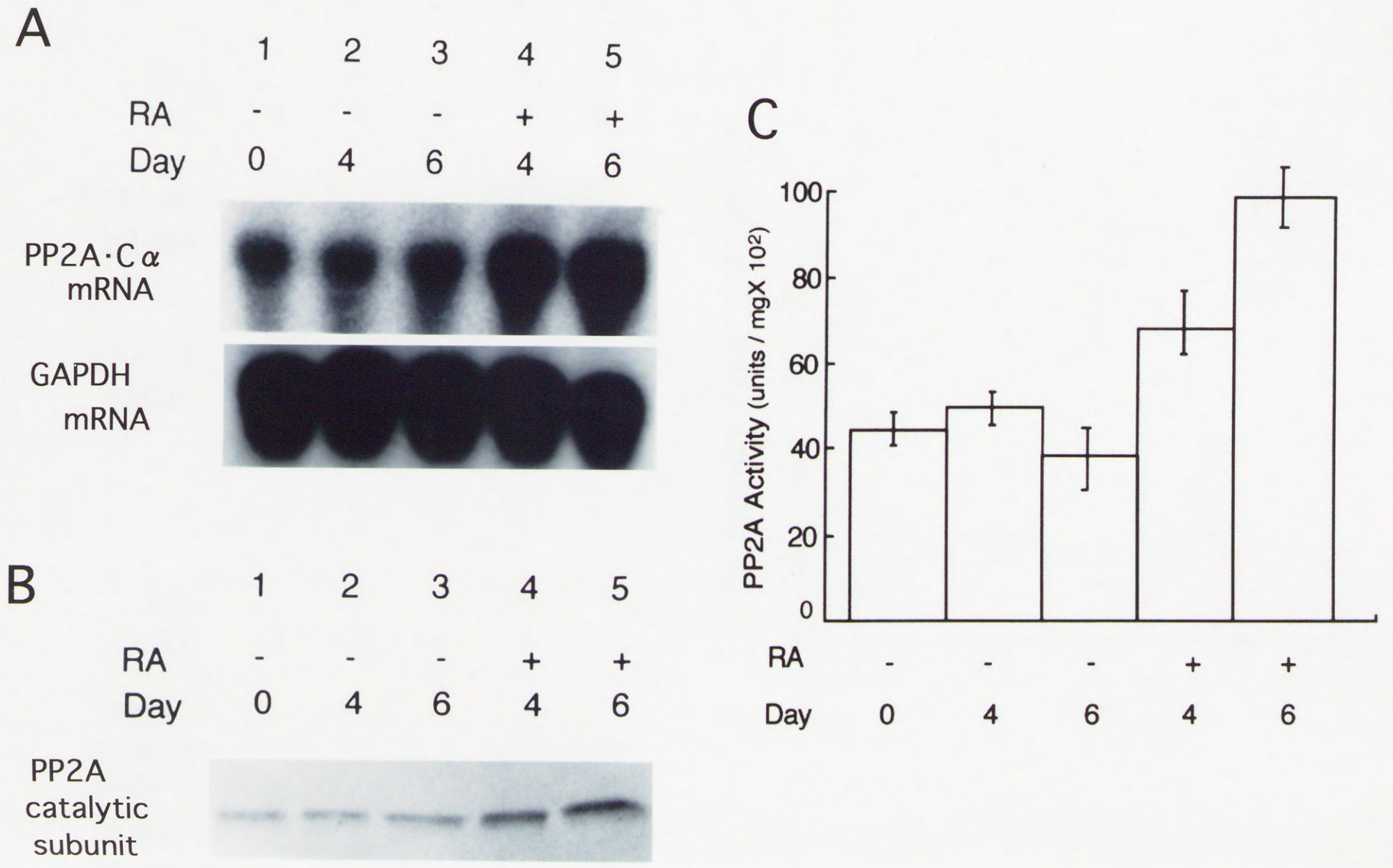
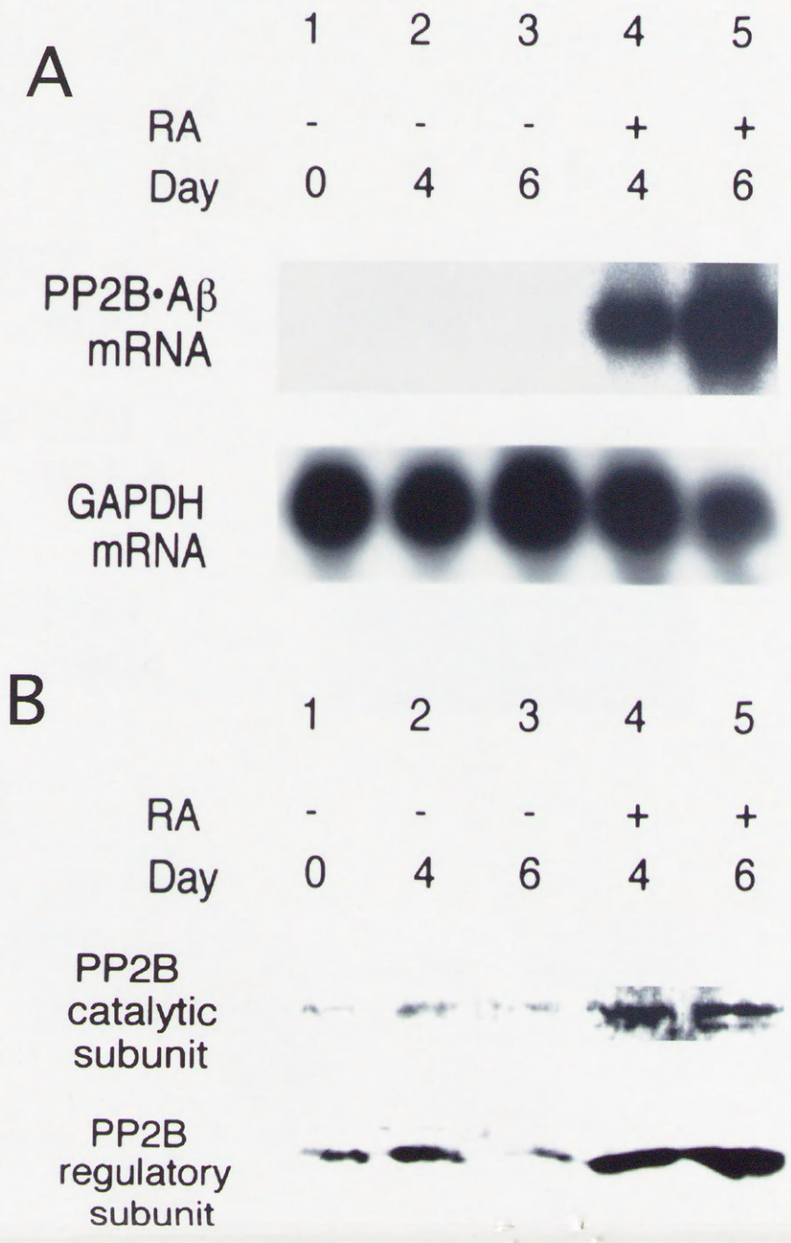

A
1 2 3 4 5
RA - - - + +
Day 0 4 6 4 6
PP2A·Cα mRNA
GAPDH mRNA
B
1 2 3 4 5
RA - - - + +
Day 0 4 6 4 6
PP2A catalytic subunit
C
PP2A Activity (units / mgX 10²)
100
80
60
40
20
0
RA - - - + +
Day 0 4 6 4 6

Fig.5


A
1 2 3 4 5
RA - - - + +
Day 0 4 6 4 6
PP2B•Aβ mRNA
GAPDH mRNA
B
1 2 3 4 5
RA - - - + +
Day 0 4 6 4 6
PP2B catalytic subunit
PP2B regulatory subunit

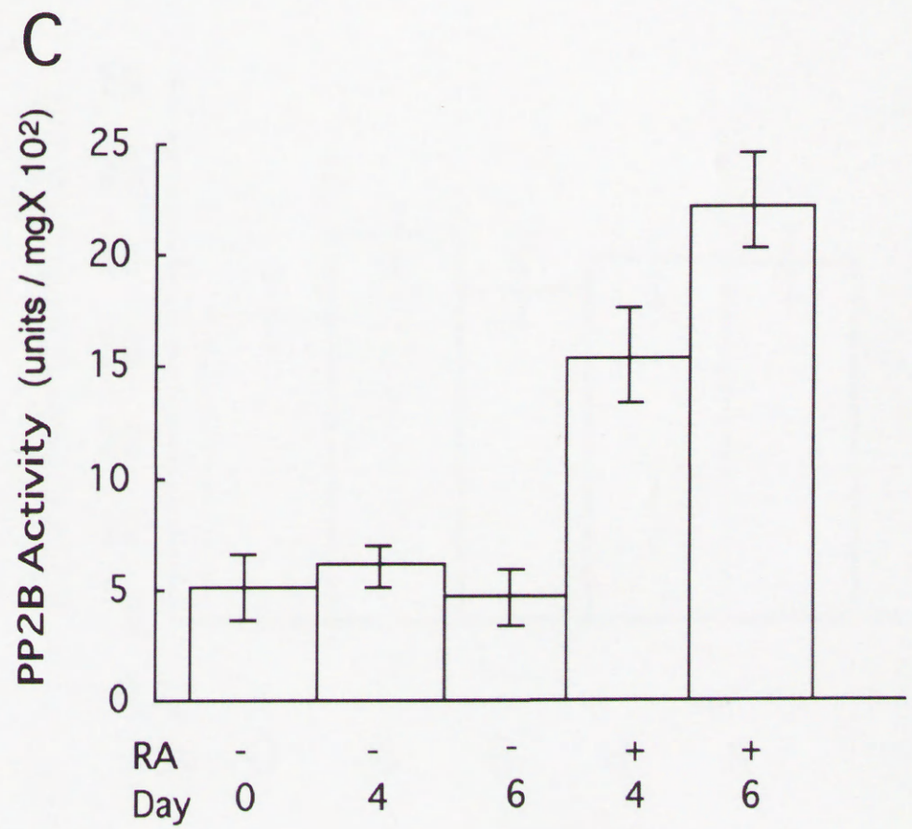
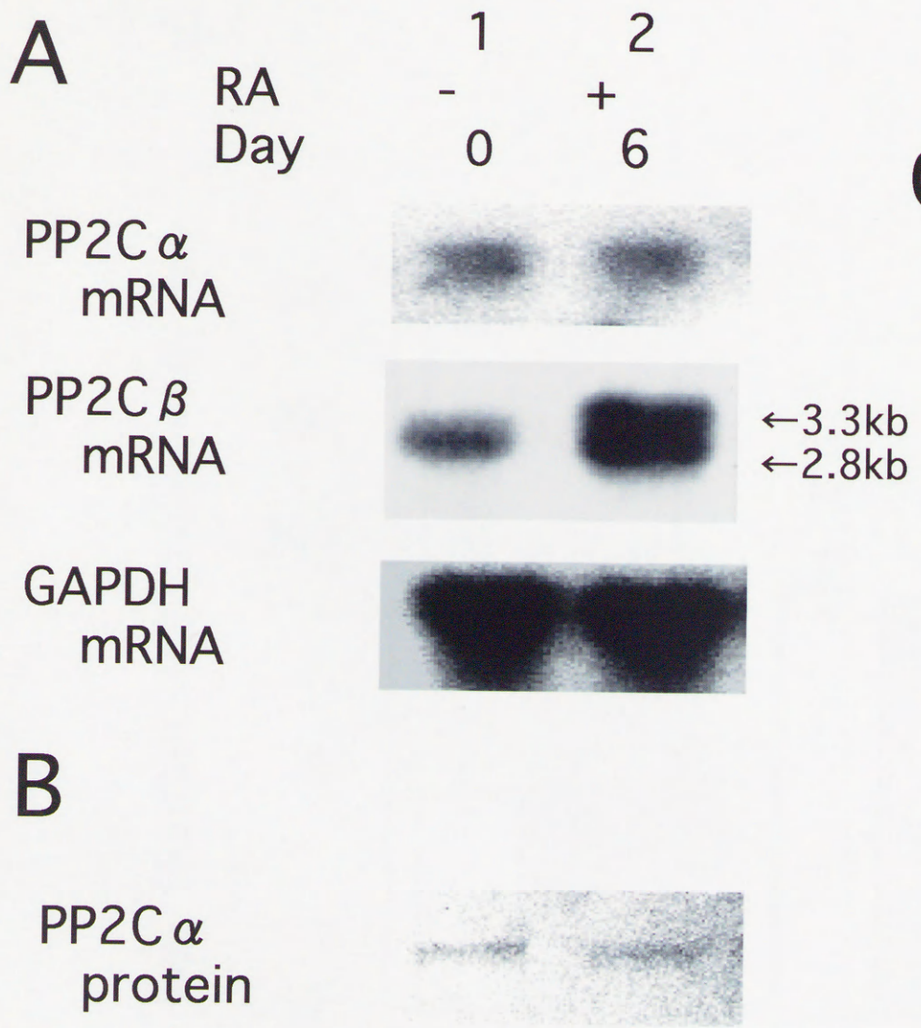

C
PP2B Activity (units / mgX 10²)
25
20
15
10
5
0
RA - - - + +
Day 0 4 6 4 6

Fig.6


A
1
2
RA
-
+
Day
0
6
PP2C α
mRNA
PP2C β
mRNA
←3.3kb
←2.8kb
GAPDH
mRNA
B
PP2C α
protein

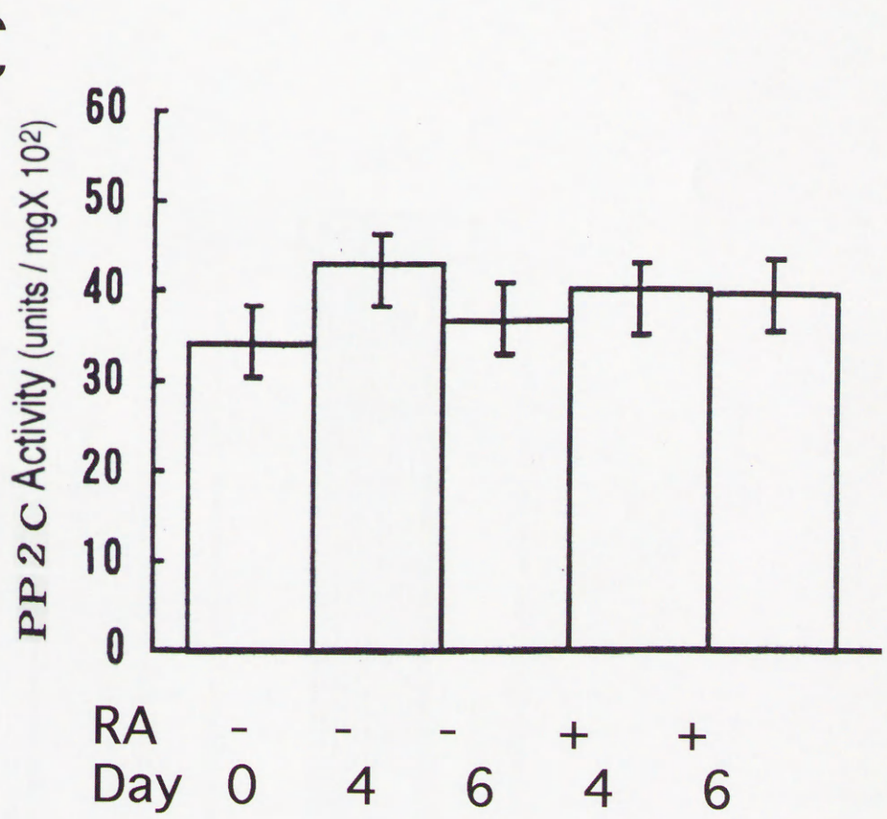

C
PP 2 C Activity (units / mgX 10²)
60
50
40
30
20
10
0
RA - - - + +
Day 0 4 6 4 6

Fig.7

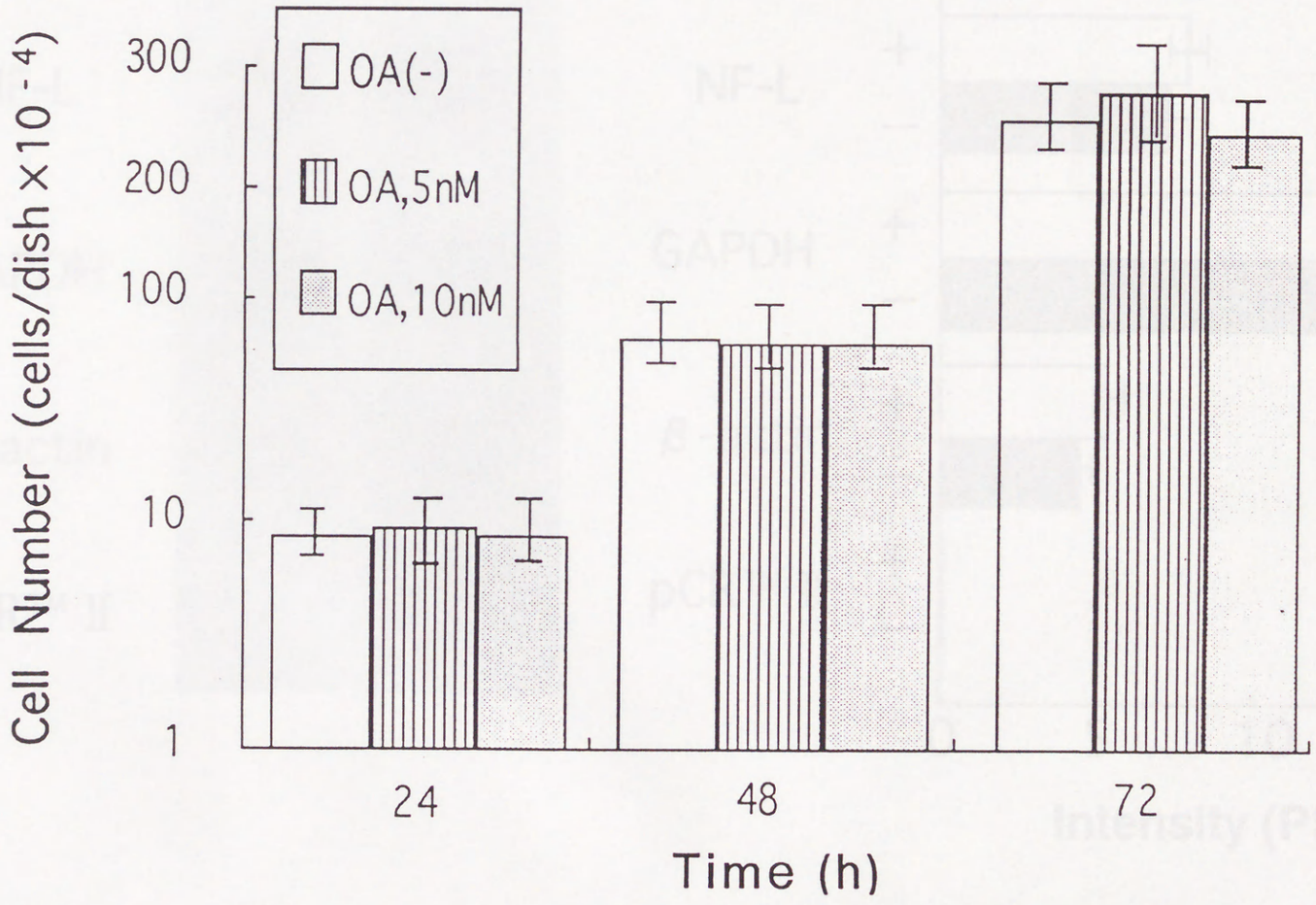

Cell Number (cells/dish ×10⁻⁴)
300
200
100
10
1
OA(-)
OA,5nM
OA,10nM
24
48
72
Time (h)

Fig.8

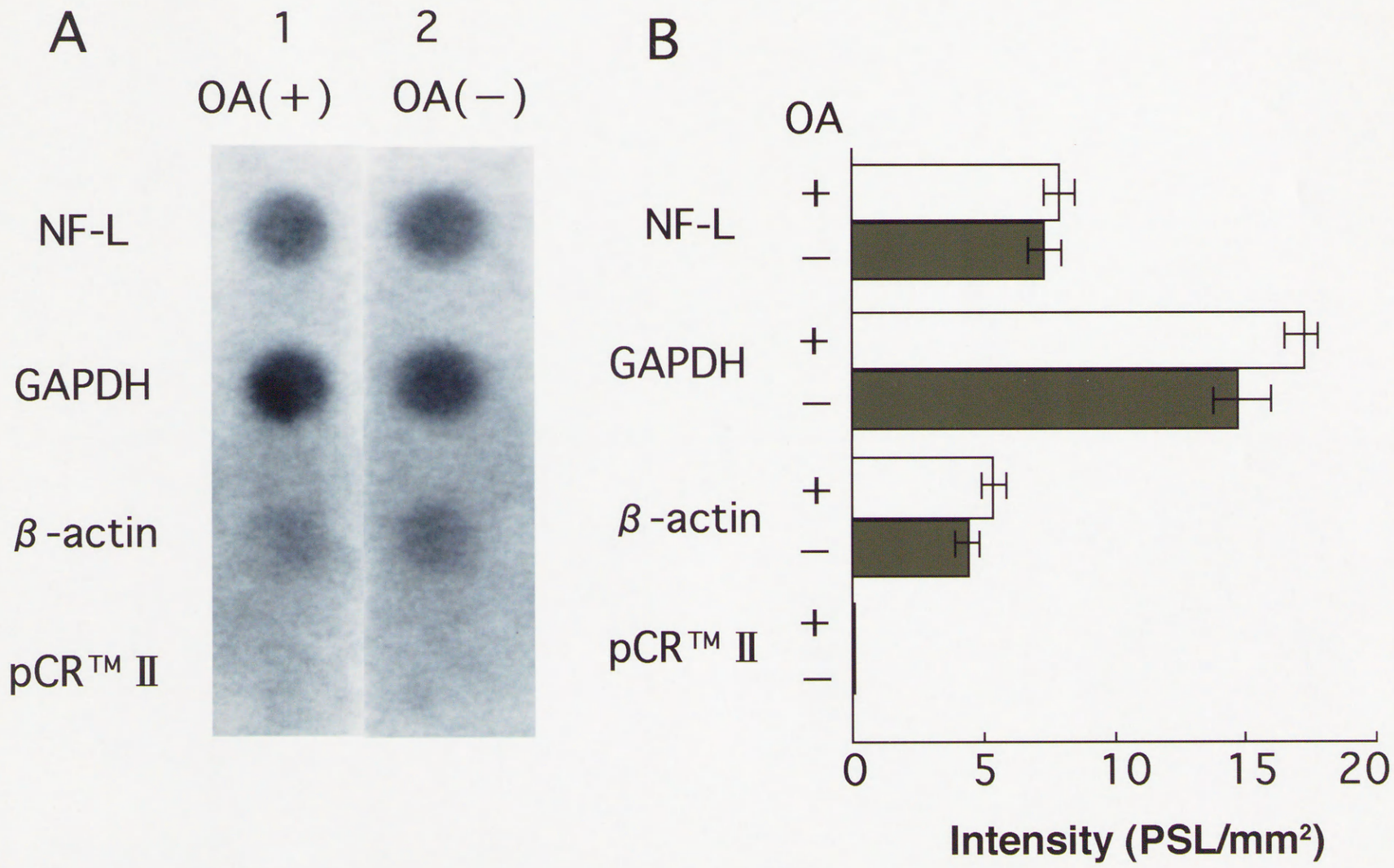
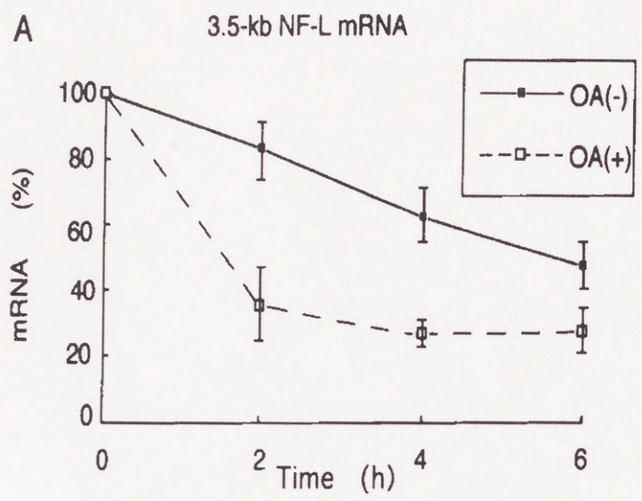
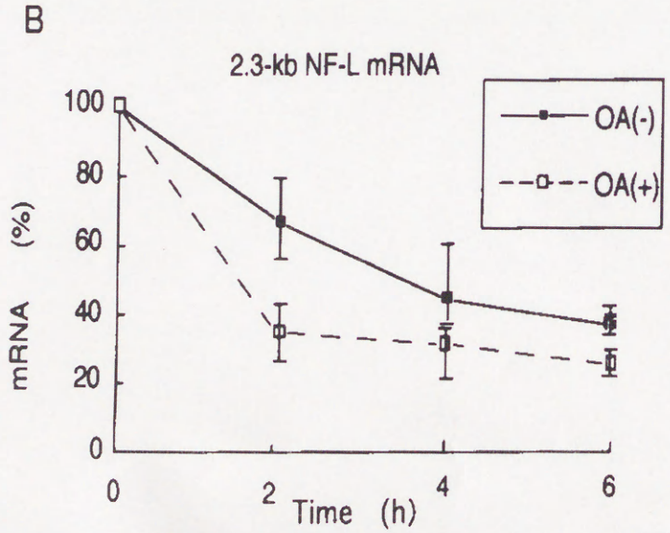

A
1
2
OA(+)
OA(−)
NF-L
GAPDH
β-actin
pCR™ II
B
OA
NF-L
GAPDH
β-actin
pCR™ II
+
−
0
5
10
15
20
Intensity (PSL/mm²)

Fig.9


A
3.5-kb NF-L mRNA
OA(-)
OA(+)
mRNA (%)
100
80
60
40
20
0
0
2
4
6
Time (h)


B
2.3-kb NF-L mRNA
OA(-)
OA(+)
mRNA (%)
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80
60
40
20
0
0
2
4
6
Time (h)

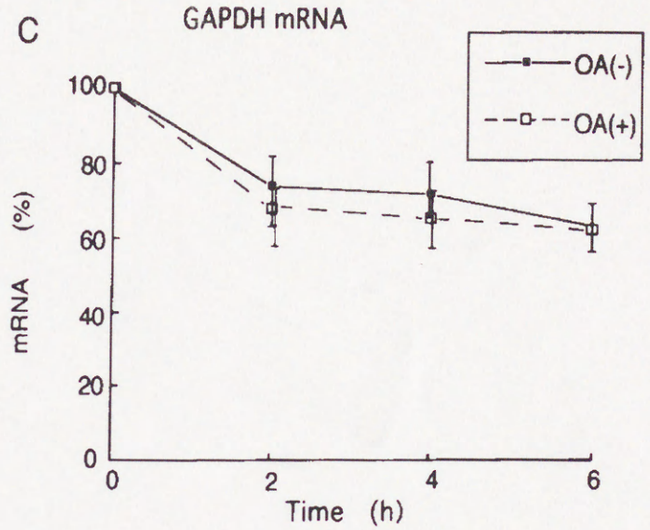
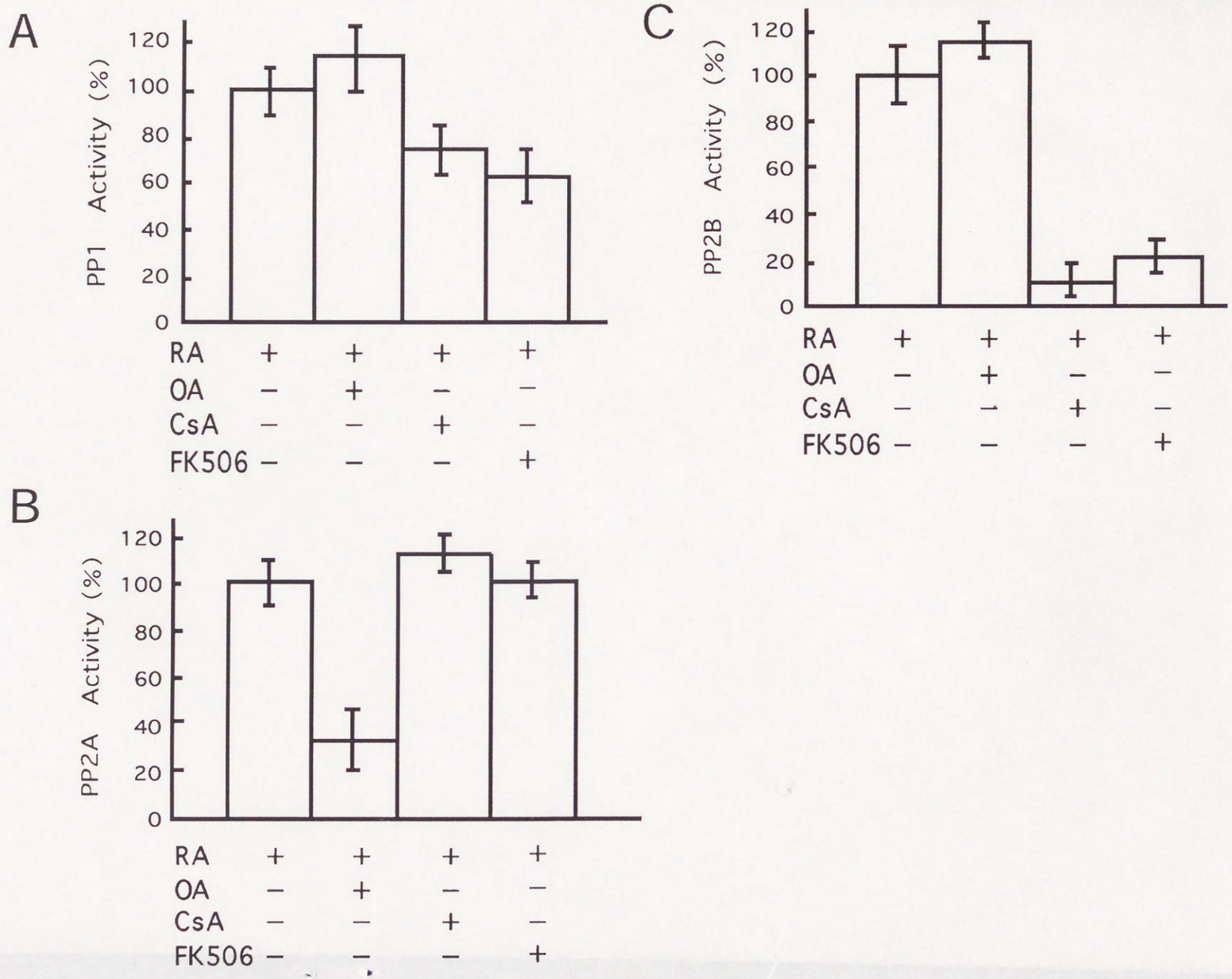
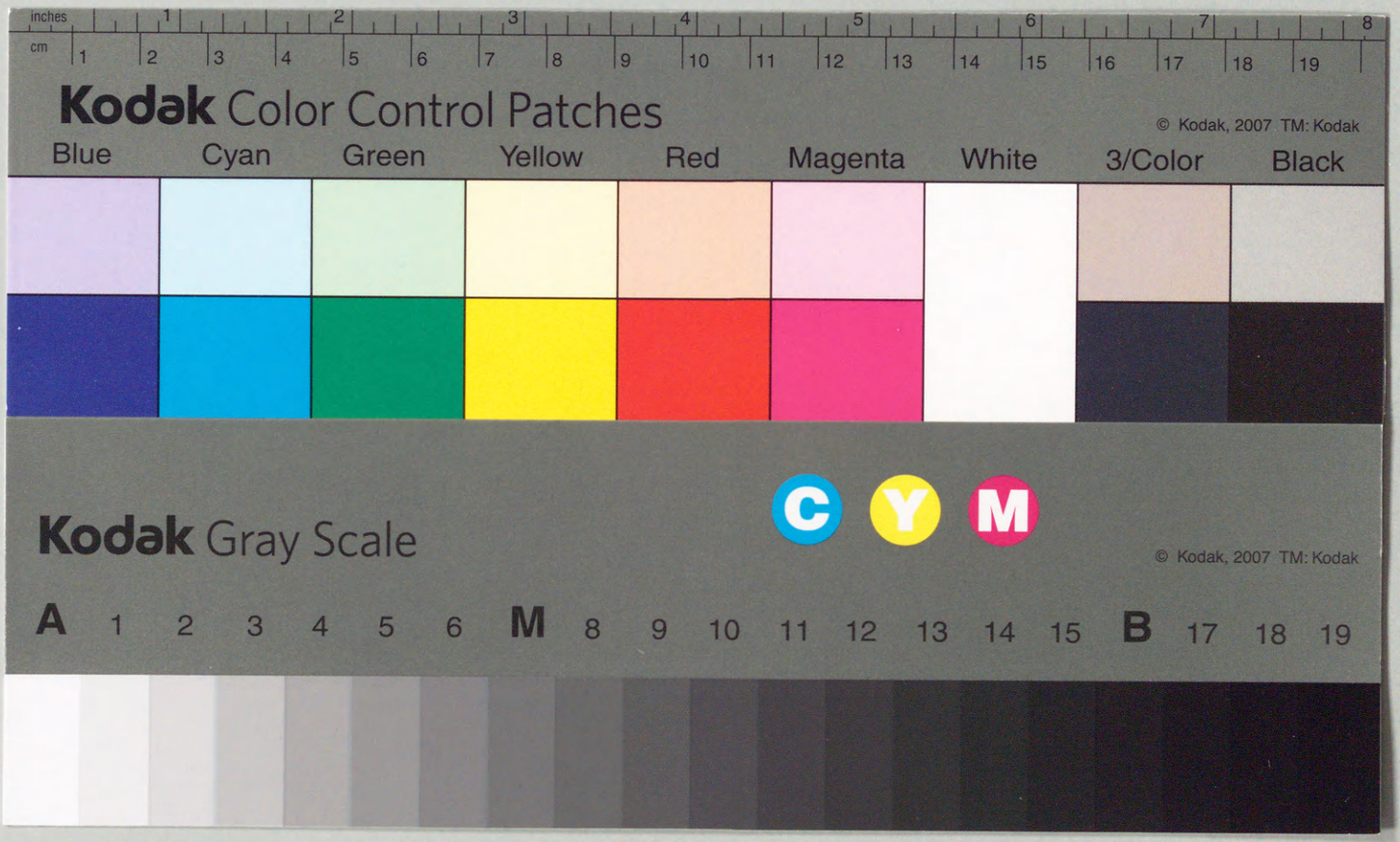

C
GAPDH mRNA
OA(-)
OA(+)
mRNA (%)
100
80
60
40
20
0
0
2
4
6
Time (h)

Fig.10


A
PP1 Activity (%)
120
100
80
60
40
20
0
RA + + + +
OA − + − −
CsA − − + −
FK506 − − − +
B
PP2A Activity (%)
120
100
80
60
40
20
0
RA + + + +
OA − + − −
CsA − − + −
FK506 − − − +
C
PP2B Activity (%)
120
100
80
60
40
20
0
RA + + + +
OA − + − −
CsA − − + −
FK506 − − − +


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19